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RESEARCH PAPER

Preclinical studies investigating the neural mechanisms involved in the co-morbidity of migraine and temporomandibular disorders: the role of CGRP

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Abstract

Background and Purpose: Temporomandibular disorders (TMD) and migraine can be co-morbid. This can be a significant factor in exacerbating and increasing the prevalence of migraine-like symptoms. However, the underlying mechanisms involved are unknown. Our objective was to investigate these neural mechanisms and the role of CGRP as a key modulator in this co-morbidity.

Experimental Approach: We combined experimental approaches using CGRP, which triggers a migraine-like response in patients, with that of masseteric muscle injection of complete Freund's adjuvant (CFA), to model myofascial TMD-like inflammation. Using validated electrophysiological methods to assess each of the above approaches independently or in combination, we examined their effects on the response properties of migraine-like dural-trigeminocervical neurons.

Key Results: Independently, in ~2/3 of animals (rats) each approach caused delayed migraine-like activation and sensitisation of dural-trigeminocervical neurons. The response to masseteric-CFA was attenuated by a selective CGRP receptor antagonist. The combination approach caused a migraine-like neuronal response in all animals tested, with somatosensory-evoked cranial hypersensitivity significantly exacerbated.

Conclusion and Implications: The data demonstrate a neuronal phenotype that translates to the exacerbated clinical co-morbid phenotype, supporting this combination approach as a relevant model to study the mechanisms involved. It provides a pathophysiological rationale for this exacerbated phenotype, strongly implicating the involvement of CGRP. The results provide support for targeting the CGRP pathway as a novel monotherapy approach for treating this co-morbid condition. This has key implications into our understanding of this co-morbid condition, as well as potentially addressing the major unmet need for novel and effective therapeutic approaches.

KEYWORDS

central sensitisation, CGRP, co-morbidity, hypersensitivity, migraine, temporomandibular disorders, trigeminovascular

Abbreviations: CFA, complete Freund's adjuvant; TMD, temporomandibular disorders; WDR, wide dynamic range.

1 | INTRODUCTION

Primary headache disorders, particularly migraine, are co-morbid with the constellation of musculoskeletal orofacial pain conditions known as temporomandibular disorders (TMD) (Ballegaard, Thede-Schmidt-Hansen, Svensson, & Jensen, 2008; Bevilaqua Grossi, Lipton, & Bigal, 2009; Goncalves et al., 2011; Goncalves, Bigal, Jales, Camparis, & Speciali, 2010). Both are highly prevalent and disabling (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017; Isong, Gansky, & Plesh, 2008; Slade et al., 2016), but together each condition can be significantly exacerbated. The major feature of this co-morbidity, particularly myofascial TMD with migraine, is that the presence of either disorder is considered a risk factor for the other, with each increasing the likelihood of the other occurring (Ballegaard et al., 2008; Goncalves et al., 2010; Goncalves et al., 2011; Goncalves, Speciali, Jales, Camparis, & Bigal, 2009; Tchivileva et al., 2017). However, this relationship goes beyond increased likelihood of co-occurrence. The presence of TMD in migraine patients is a significant factor in increasing headache severity and the prevalence, with migraine and chronic daily headache becoming far more likely (Goncalves et al., 2011). This negatively impacts on their therapeutic management (Fernandes et al., 2013; Franco et al., 2010; Goncalves et al., 2010; Tchivileva et al., 2017). Furthermore, evidence suggests that the presence of TMD in migraine patients is a significant risk factor for migraine becoming chronic (Bevilaqua Grossi et al., 2009; Bevilaqua-Grossi et al., 2010; Bigal et al., 2008). Together, this underscores the impact of TMD in the headache population and the relevance of this co-morbidity as a serious but often overlooked health concern.

The underlying neural mechanisms of this co-morbidity and the exacerbated headache phenotype that TMD creates are unknown, and largely unstudied, limited somewhat by a lack of preclinical approaches. To some extent primary headaches, such as migraine, and TMD share the trigeminal system in their mechanisms. CGRP, a neuropeptide thought to be integral in causing migraine, is also involved in TMD pathogenesis and the cranial hypersensitivity that occurs, and it is proposed as a target for TMD treatment (Cady, Glenn, Smith, & Durham, 2011; Romero-Reyes, Pardi, & Akerman, 2015; Shu et al., 2020). Here, we provide the framework to study this co-morbidity, postulating that there is an overlap in the physiology between these disorders, with the additional common molecular link through CGRP which drives migraine-like dural-trigeminovascular neuronal activation and sensitisation. This ultimately leads to neuronal hypersensitivity, which underlies the exacerbated pain phenotype found when these disorders are co-morbid. We used an established electrophysiological technique to record migraine-like dural-responsive central trigeminocervical neurons, utilizing migraine-like, TMD-like and then a combination of these preclinical paradigms, to determine whether (i) V3-masseteric muscle inflammatory pain (TMD-like) produces V1-intracranial dural and extracranial cutaneous (migraine-like) neuronal responses, (ii) whether this exacerbates and/or increases the likelihood of migraine-like responses, when the TMD-like approach is combined with a migraine provocative and (iii) whether CGRP is an important mediator in this response. Further, the data provide support for this combination preclinical approach as a relevant model to study pathogenic mechanisms and therefore has the potential to screen possible therapeutic approaches for this co-morbidity.

What is already known

- Migraine and temporomandibular disorders are co-morbid disorders.
- CGRP is strongly implicated in migraine mechanisms and potentially also temporomandibular disorders.

What does this study add

- Preclinically, a temporomandibular disorders-like approach mediates a migraine-like trigeminovascular neuronal response, which involves CGRP.
- Modelling both pathophysiologies together causes an exacerbated migraine-like trigeminovascular neuronal response, involving CGRP.

What is the clinical significance

- This confirms the symptomatic phenotype of this co-morbidity and as a model to study its mechanisms.
- This implicates CGRP in the mechanisms involved and as a monotherapy for this co-morbid state.

2 | METHODS

All experiments were conducted in compliance of a research protocol approved by NYU or University of Maryland Baltimore Institutional Animal Care and Use Committee and those of the Committee for Research and Ethical Issues of IASP (Zimmermann, 1983). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

2.1 | Animals-experimental design

We used adult male Sprague–Dawley rats (250–390 g, Charles River, MA, USA) throughout these studies. Using male rats in preclinical migraine studies has proven a reliable and translational approach to study migraine mechanisms and predict the efficacy of

treatments, as well as using validated preclinical models which have a significant overlap to the human migraine pain pathway. Because of this, it allows direct comparison with our and previous data by other authors. Furthermore, it allows us to circumvent the complexity of oestrus in animal migraine models, which is still not fully understand. Prior to the study, rats were housed in temperature and light controlled rooms for at least 7 days before use, with access to food and water *ad libitum*. At the end of each experiment, all animals were killed using 200 mg·kg^{-1} Euthasol (i.v.; **pentobarbitone**). We used CGRP infusion (i.v.) as our migraine-like model, known to trigger migraine-like headache in a significant proportion of patients (Lassen et al., 2002). Masseteric muscle injection of complete Freund's adjuvant (CFA) was used to model TMD-like inflammatory (myositis) pain (Asgar et al., 2015; Romero-Reyes et al., 2013). These approaches were used independently and also in tandem for the co-morbid model,in separate groups of rats. We used a selective **CGRP receptor** antagonist to dissect CGRP's role in TMD-like responses.

2.2 | Animal preparation and electrophysiological recording

The surgical preparation, physiological monitoring and electrophysiological recording methods and analyses are the same as those reported in detail previously (Akerman et al., 2019; Akerman & Goadsby, 2015; Akerman, Holland, Lasalandra, & Goadsby, 2010). Rats were anaesthetised with sodium pentobarbitone (Nembutal, 70 mg·kg^{-1}, intraperitoneal) and maintained with **propofol** (PropoFlo™ 15-25 mg·kg^{-1}·h^{-1}, intravenous infusion) and prepared for electrophysiological extracellular recording of dural-responsive neurons in the trigeminocervical complex (TCC), using aseptic techniques. BP, body temperature (36.5–37°C) were monitored and kept within physiological limits. Rats were artificially ventilated with oxygen enriched air, and expired CO_2 continuously monitored and ventilation adjusted as required to maintain normal physiological limits (3.5–4.5%). During electrophysiological recording animals were paralysed with a neuromuscular blocker; **pancuronium bromide**

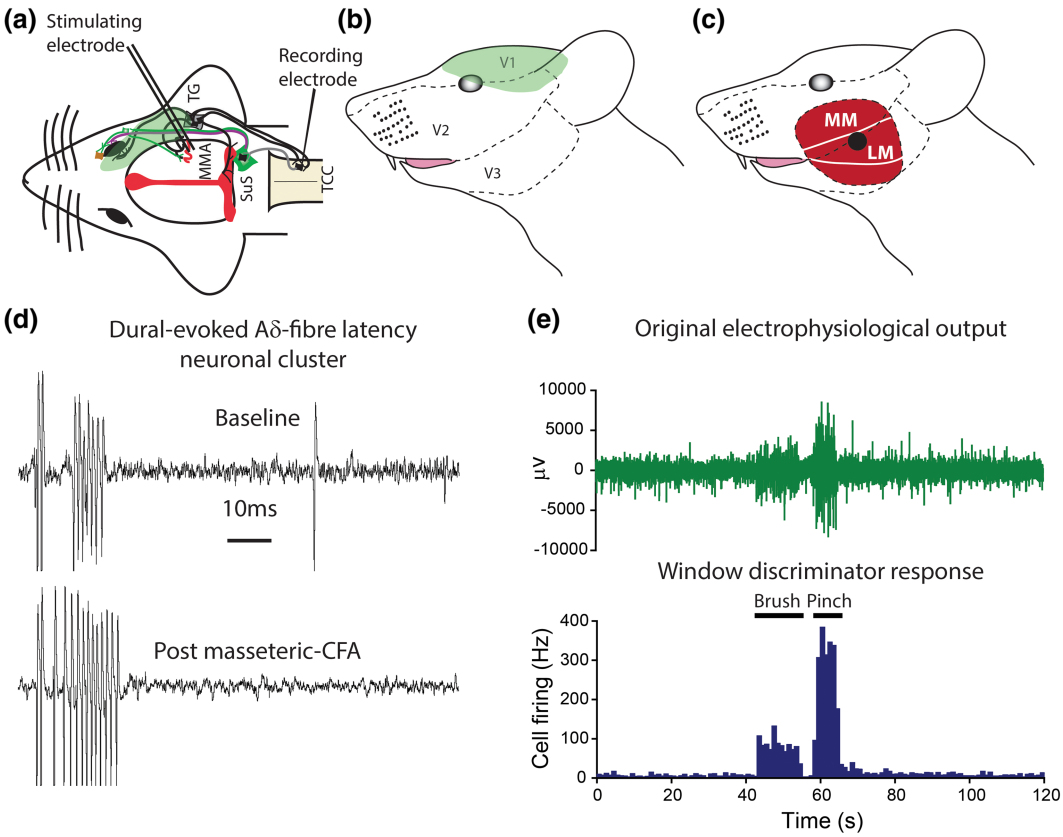

FIGURE 1 Experimental set-up for electrophysiological studies. (a) Neurons of the trigeminocervical complex (TCC) were recorded in response to electrical stimulation of the trigeminal innervation of the dural meninges, and innocuous and noxious stimulation of the cutaneous facial receptive field (shaded area). (b) Somatotopic representation of the trigeminal territories for receptive field characterisation and an example receptive field region (shaded area); V1, ophthalmic; V2, maxillary; V3, mandibular. (c) Area of masseteric musculature for CFA injection, in the V3 region; MM, medial masseter; LM, lateral masseter. (d) Original tracing of a single sweep (stimulus) of a reproducible, dural-evoked neuronal cluster classified as receiving Aδ-fibre input (<20 ms latency, "fast" neuronal responses), prior to any treatment (baseline—upper plot). Lower plot is an example after administration of CFA (50 μl), with the number of Aδ-fibre latency action potentials spikes increased, indicative of a hypersensitive neuronal response. (e) Original example of the electrophysiological neuronal response to innocuous brush and noxious pinch of the cutaneous V1 receptive field. Top panel is original electrophysiological output; bottom panel is responses that cross the window discriminator

(Pavulon®, Organon) 0.4 mg initially and maintained with 0.2 mg every 35 min. A sufficient depth of anaesthesia was judged by the absence of paw withdrawal and corneal blink reflex and during muscular paralysis by fluctuations of BP and changes in expired CO_2.

2.3 | Characterisation of neurons

This has been described in detail previously (Akerman et al., 2010; Akerman et al., 2019; Akerman & Romero-Reyes, 2017). Briefly, a tungsten recording electrode (0.5–1 MΩ, tip diameter 0.5 μm) was advanced into the trigeminocervical complex at 5 μm increments (Figure 1a). Neuronal responses were characterized for their cutaneous and deep muscle receptive field, assessing noxious and innocuous responses through all three trigeminal territories (Figure 1b). Neurons sensitive within the ophthalmic V1 facial dermatome were identified, responses were then tested for convergent nociceptive input from the trigeminal innervation of the dura mater, using square-wave electrical stimulation (100–200 μs pulse, 0.25 Hz, and 8–15 V), and electrode position and electrical parameters optimized for the most robust neuronal response. These parameters are capable of activating both Aδ ("fast" responses; Figure 1d) and C-fibre neurons ("slow" responses), based on the approximate conduction velocities (Millan, 1999) and the distance from the dural stimulation site to trigeminocervical complex recording site (30–40 mm) (Akerman & Goadsby, 2015). The V1 facial dermatome receptive field characterisation consisted of 10 brush strokes applied to the facial receptive field above the eyes over 7–8 s for the innocuous response using a cotton tip applicator, and pinch with forceps for 4 s that was painful when applied to humans, for the noxious response (Figure 1b,e). Spontaneous activity (spikes per second, Hz) was recorded throughout and measures for analysis taken for 300 s preceding the dural stimulation (Figure 1e). Post- and peri-stimulus time histograms of neural activity were displayed and analysed using Spike2 v8. These methods have been proven to reliably explore putative mechanisms that contribute to migraine,and other headaches, and predict both abortive and preventive therapeutics (Akerman et al., 2010; Akerman et al., 2019; Akerman & Goadsby, 2015).

2.4 | Materials

Rat-CGRP (Tocris Bioscience, USA) was dissolved in 0.9% NaCl (saline solution) aliquoted and frozen until required. On the day of the experiment, it was made up into a solution with saline and given as an intravenous infusion (300 $ng \cdot kg^{-1} \cdot min^{-1}$ for 20 min). CFA (each ml contains 1 mg heat killed and dried mycobacterium *Mycobacterium tuberculosis*, with 0.85 ml paraffin oil and 0.15 ml mannide monooleate, Sigma-Aldrich, USA) was given intramuscularly (50 μl) into the V3 masseteric musculature (Figure 1c). CFA was given 5 min prior to the beginning of CGRP infusion in the co-morbid experimental group. **BIBN4096** (Tocris Bioscience, 900 $\mu g \cdot kg^{-1}$) (Akerman, Holland, Summ, Lasalandra, & Goadsby, 2012; Koulchitsky, Fischer, & Messlinger, 2009) was dissolved in a 1:20 solution of DMSO and 0.9% NaCl, and made up fresh each day and given 5 min prior to CFA injection. Saline was used as the vehicle control throughout.

2.5 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Overall, the studies were designed to generate equal group sizes and animals were assigned to experimental groups randomly and were operator blinded with respect to chemical intervention and during analysis. The animals per group is based on published data and previous experience and sample size calculations (power analysis) that typically sees difference in means of 25–30% (*SD* = 15–20%) with a two-sided alpha of 0.05 and power of 80–90%, calculated using Gpower software. This typically requires a sample size of 12–15 animals to characterize potentially differential neuronal responses over 3 h and 10–12 animals in response to drug treatments. Based on our previous studies characterizing the response of nitroglycerin (Akerman et al., 2019), not every animal responded. Therefore, to appropriately power the study, a larger group size was used for the CGRP group, n = 15. For the remaining studies n = 12 was considered appropriate. Two rats were excluded from the co-morbid CGRP-CFA group as the recording cell was lost during the recording. This was caused by movement of the spinal cord resulting in change in the recording site, and it was not possible to recover the cell and complete the full recording window for these experiments. Throughout, neuronal responses within the C-fibre range were only observed in a subset of all animals studied, compared to every animal within the Aδ-fibre range; hence these group sizes are smaller and vary. Further, as some groups were split into "responders" and "non-responders," on some occasions, we report data for groups n < 5, purely for the purposes of demonstrating this split. While relevant to the study, we consider these data exploratory based on the limited n, with no statistical analysis undertaken. Overall, these group sizes represent the number of independent values and that statistical analysis was done using these independent values (i.e. no technical replicates).

The exact latency of neuronal discharges was established separately in each experiment with this latency window used throughout. The data collected from post-stimulus histograms represent the number of action potential spikes that fired within a latency window per stimulation, averaged over 20 stimulations (sweeps; action potential spikes/sweep). Cutaneous receptive field responses and ongoing spontaneous neuronal activity are measured in cell firings per second (Hz). A neuron was considered activated using the critical ratio test (Nagler, Conforti, & Feldman, 1973), which in effect implies that greater than 30% change from baseline firing at least two time points is considered significant, and 10–30% change unclassified, over the course of the study. A neuron was considered sensitised if it exhibited enhanced responses to at least three of five parameters; dural electrical stimulation, brushing, or pinching of the cutaneous facial receptive

field; or expansion of dural or cutaneous facial receptive field, based on Melo-Carrillo, Noseda, et al. (2017).

For graphical purposes, data are presented as mean ± SEM, and all statistical analyses were conducted on raw data and tested for homogeneity of sample variance (where appropriate) using Levene's test. For all groups, data were assessed through all data points over the first 60 min, and the average response across data points is taken every 30 min over 3 h. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$. Statistical analyses were performed using ANOVA for repeated measures to determine the time course of significant drug intervention, using a 95% confidence interval. Where required, if Mauchly's test of sphericity was violated, we made appropriate corrections to degrees of freedom according to Greenhouse–Geisser. Post hoc analysis was only conducted following a significant ANOVA test to protect from Type I errors. Student's paired two-tailed *t*-test for post hoc analysis was used to test for the time points of significance, using the average of the two or three baselines for comparison, using the criteria of Bonferroni correction for multiple testing. A two-way between subjects' mixed design repeated measures ANOVA was used to compare responses between groups. If the test residuals were not normally distributed, we performed equivalent non-parametric tests using Friedman's ANOVA and post hoc Wilcoxon's test, where applicable. Statistical significance was set at $P < 0.05$ (IBM-SPSS 25.0).

2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Neuronal identification and properties

Electrophysiological recordings were made from 51 neuronal clusters (one cluster per animal) that responded to electrical stimulation of the trigeminal afferent innervation of the dura mater and with cutaneous facial receptor fields that included the first division (V1) of the trigeminal nerve and on occasions also the second (V2) and third (V3) division (Figure 1a–e). Of these 51, 42 (40 wide dynamic range [WDR], 1 nociceptive-specific, and 1 low-threshold) exhibited a combination of either reproducible burst (action potentials in close proximity of each other) of discharges at 3–20 ms (Aδ-fibre) or 3–30 ms (both Aδ and C-fibre), with also unitary (single isolated action potentials) discharges at 20–90 ms (C-fibre), which were classified as receiving both Aδ and C-fibre inputs (both "fast" and "slow" responses). Neuronal bursts ($n = 9$; all WDR) exhibiting only early discharge responses at 3–20 ms (Aδ-fibre), were classified as receiving only Aδ-fibre inputs ("fast" responses, Figure 1a,b). Neurons were located in mainly nociceptive-specific superficial (laminae I-II) and deeper layers (laminae V and VI) of the dorsal horn of the trigeminocervical complex at range of depth of 190–975 μm, based on readings from the piezoelectric motor controller used to lower the electrode into the spinal cord, which indicates distance travelled from the surface of the spinal cord.

3.2 | Summary of baseline responses

Through all animals/groups studied, the average baseline spontaneous firing rate was 21.1 ± 1.6 Hz, and there was no significant difference across the different groups. There was also no significant difference at baseline between all groups for dural-evoked Aδ-fibre units, or unitary C-fibre discharges, with an average baseline of 8.7 ± 0.2 and 1.2 ± 0.14 action potential spikes per sweep, respectively. There was also no significant difference at baseline between groups in response to either innocuous or noxious somatosensory stimulation of the facial cutaneous receptive field. The physiological characteristics of this neuronal population are similar to previous studies (Akerman et al., 2019; Akerman & Goadsby, 2015).

3.3 | CGRP triggers a migraine-like phenotype in a significant subset of dural-responsive trigeminocervical neurons

Overall CGRP (300 $ng \cdot kg^{-1} \cdot min^{-1}$ for 20 min, i.v.) caused a significant increase in spontaneous trigeminal neuronal firing over 3 h ($n = 15$) but not over the first 60 min. Specifically, change was significant after 90 min, which remained so for 150 min. Based on the critical ratio test (Nagler et al., 1973) 6/15 neuronal populations in rats did not increase activity above 30% across all time points ("non-responders"), and there was no significant change over 3 h. In the 9/15 neuronal populations ("responders") that reached the critical ratio, there was a significant increase in firing over the 3 h, specifically after 60 min that was maintained through 3 h (summarized in Table 1 and Figure 2a,d). In the same neurons, there was a significant increase in the neuronal response to dural stimulation for all "fast" neuronal discharges. This was significant after 120 min and was maintained beyond 3 h. Analysing the "non-responders" and "responders" separately, the "non-responders" did not produce a significant increase in dural-evoked neuronal responses. The "responders" caused a significant increase in dural-evoked neuronal firing, specifically after 90 min that was maintained through 3 h (summarized in Table 1 and Figure 2b). There was no effect of CGRP on dural-evoked unitary discharges within the "slow" C-fibre range (Figure 2c).

In 7/9 neuronal clusters that responded to CGRP, there was expansion of the cutaneous facial receptive field (Figure 2e,f). There was also a hypersensitive neuronal response to cutaneous facial stimulation, with increased firing in response to innocuous brush and noxious pinch after 90 min. There was no change over the 3 h for the "non-responders". However, there was a significant increase with the

TABLE 1 Summary of trigeminocervical neuronal data over 3 h, with responses at baseline and when significantly increased, in response to CGRP (mean ± SEM)

	Spontaneous activity (Hz)	
CGRP grouped data	Baseline	24.2 ± 4.1; $F_{2.9,40.1}$ = 4.2, P = 0.012, n= 15
	Post CGRP 90 min	32.8 ± 5.4; t_{14} = 2.9, P = 0.011*
CGRP "responders"	Baseline	23.2 ± 5.3; $F_{2.6,20.5}$ = 6.4, P = 0.004, n = 9
	Post CGRP 60 min	37.2 ± 7.3; t_8 = 3.8, P = 0.008*
	Dural-evoked "fast" neuronal responses (AP/Stim)	
CGRP grouped data	Baseline	8.9 ± 0.5; $F_{3.0,42.2}$ = 6.0, P = 0.002
	Post CGRP 120 min	10.2 ± 0.8; t_{14} = 6.4, P = 0.004*
CGRP "responders"	Baseline	9.2 ± 0.4; $F_{6,48}$ = 10.4, P < 0.05
	Post CGRP 90 min	10.4 ± 0.7: t_8 = 3.4, P = 0.009*
	Dural-evoked "slow" neuronal responses (AP/Stim)	
CGRP grouped data	Baseline	1.8 ± 0.4: $F_{6,60}$ = 0.8, P = 0.48, n = 11

Note: AP/Stim = number of action potential spikes per stimulation.
*P < 0.05 significance from baseline.

"responders" to innocuous brush and noxious pinch, both of which were significant after 45 min that was maintained through 3 h (Figure 2g,h).

3.4 | TMD-like (V3) pain triggers a migraine-like phenotype in a significant subset of dural-responsive trigeminocervical neurons

Complete Freund's adjuvant (CFA; 50 μl, i.m.) injection into the V3-masseteric musculature caused a significant increase in spontaneous firing of dural-responsive trigeminocervical neurons (n = 12). This response was significant after 60 min, returning to baseline levels by 150 min. Again, these neuronal populations were grouped into "responders" (8/12) and "non-responders" (4/12); a similar ratio to CGRP responders. The "non-responders" were considered to have not changed over time, although statistical analyses could not be performed with n < 5 and thus considered exploratory only. However, there was a significant increase in firing of the "responders." This was significant after 60 min and only returned to baseline at 3 h (summarized in Table 2 and Figure 3a,d). This change manifested as a significant increase in neuronal responses to dural stimulation for all "fast" neuronal discharges. This was significant after 90 min and was maintained beyond 3 h. In the absence of statistical testing the "non-responders" showed a similar trend to "responders," which showed a significant difference over time, specifically after 90 min but was maintained through 3 h. There was also a significant hypersensitive neuronal response of dural-evoked unitary discharges within the "slow" C-fibre range, which was significant after 90 min but not sustained beyond 150 min. There was insufficient data to test the "non-responders," although there appears to be little change, whereas the "responders" did show a significant change over 3 h (n = 7), specifically after 60 min that was not significant beyond 150 min (all data summarized in Table 2 and Figure 3b,c).

In 6/8 neuronal clusters that responded to CFA, there was expansion of the cutaneous facial receptive field (Figure 3e,f). This was not restricted to only those rats whose original receptive field overlapped across V1, through V2 and V3. There was also a hypersensitive neuronal response to cutaneous facial stimulation, with increased firing in response to innocuous brush and noxious pinch. There was a significant increase with the "responders" to innocuous brush and noxious pinch, which was significant after 45 min and maintained through 3 h (Figure 3g,h).

3.5 | Blocking the CGRP receptor pathway prevents the migraine-like phenotype in TMD-like dural-responsive trigeminocervical neurons

To confirm the role of CGRP in the CFA-mediated effects, we also used a CGRP receptor antagonist, BIBN4096 (900 $\mu g \cdot kg^{-1}$, i.v.), given prior to CFA masseter muscle injection. Despite a moderate trend for an increase in spontaneous trigeminal firing there was no significant change after CFA over 3 h (n = 12; Figure 4a). Indeed, when compared to the CFA-alone group, there was a significant difference between these groups. Only 4/12 neuronal populations were "responders," suggesting that the CGRP receptor antagonist blocks the effects of CFA in at least half, but not all cases. There was also no change in neuronal responses to dural stimulation for all "fast" neuronal discharges (Figure 4b) or for unitary discharges within the "slow" C-fibre range (n = 8; Figure 4c). Further, there was no expansion of facial receptive fields (examples in Figure 4d) or change in neuronal responses to cutaneous facial stimulation, either innocuous brush (Figure 4e) or noxious pinch (Figure 4f).

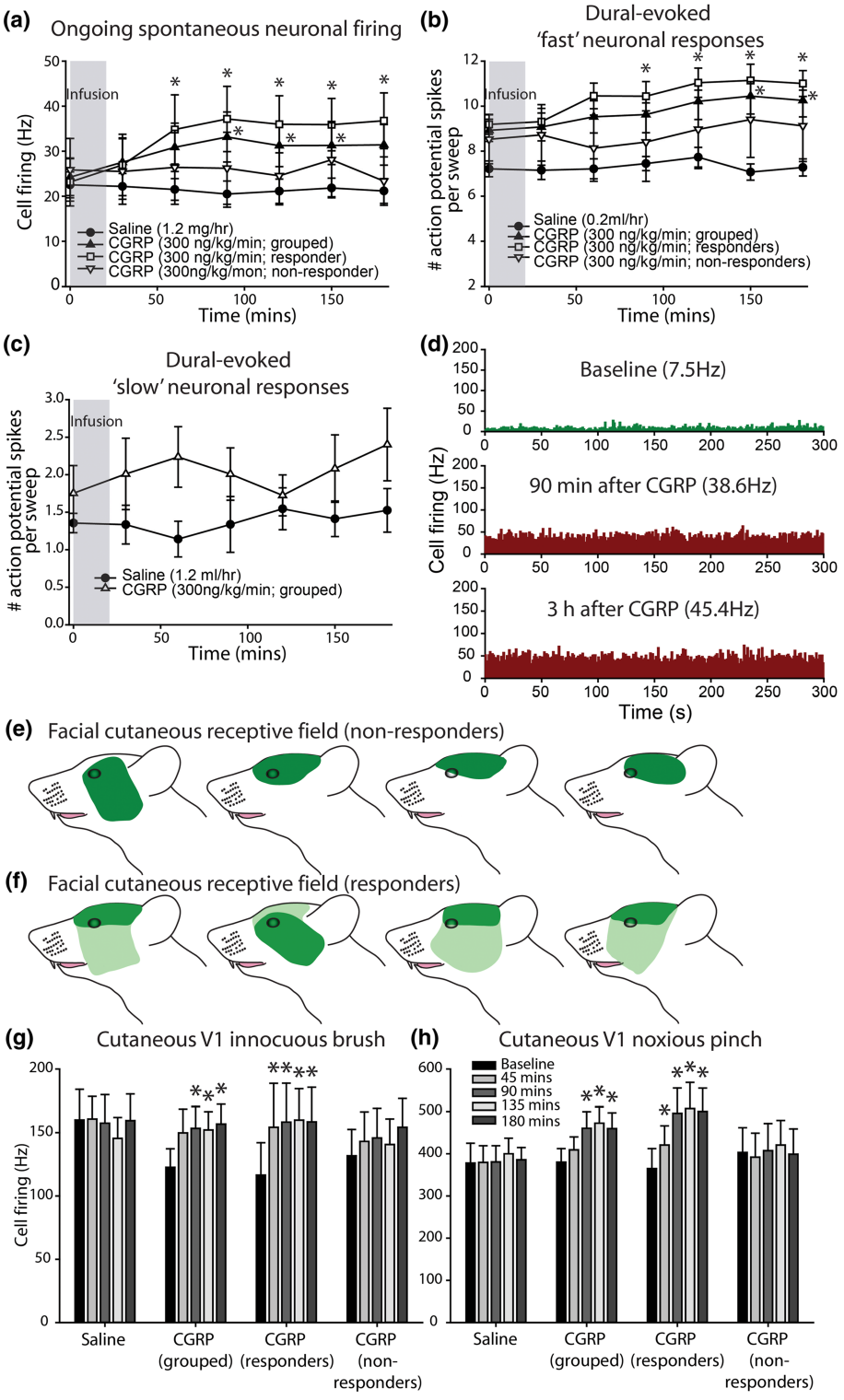

FIGURE 2 CGRP mediates delayed activation of central trigeminocervical neurons and neuronal hypersensitivity to cranial somatosensory stimulation. Time course changes in (a) spontaneous trigeminocervical neuronal firing (action potentials per second [Hz]), (b) intracranial dural-evoked "fast" neuronal responses (3–20 ms or 3–30 ms range) and (c) unitary discharges within only the "slow" C-fibre latency range (20–80 ms) in response to CGRP (300 ng·kg^{-1}·min^{-1} for 20 min, i.v., n = 15) infusion. The data are expressed as mean ± SEM. Further, the data are grouped into all data (grouped), and those considered "responders" or "non-responders" to CGRP. (d) Representative peristimulus time histograms from a single animal demonstrating ongoing trigeminocervical neuronal firing before CGRP, and at 90 min and 3 h post infusion, in a "responder" animal. The numbers indicate the mean firing (spikes per second [Hz]) over the displayed time period, green neuronal firing indicates baseline and no change in responses, red neuronal firing indicates a significant increase in neuronal firing. Overall, both the grouped and "responder" data showed a delayed increase in ongoing trigeminal neuronal firing and hypersensitivity of "fast" neuronal responses to dural-stimulation after CGRP ($^{*}P < 0.05$ compared to baseline). In the "non-responders" there was no change and there was no effect of any group for "slow" neuronal responses. Examples of cutaneous receptive fields in rats that were either (e) non-responders (no expansion) or (f) responders (expanded) to CGRP. Dark green represents the original receptive field, and light green is the expanded receptive field (including darker green region). Response magnitude to (g) innocuous and (h) noxious cutaneous stimulation of facial receptive field after CGRP. Only "grouped" and "responders" showed a delayed hypersensitive neuronal response to cutaneous stimulation after CGRP. $^{*}P < 0.05$ represents a statistical significance compared to baseline

TABLE 2 Summary of trigeminocervical neuronal data over 3 h, with responses at baseline and when significantly increased, in response to masseteric-CFA (mean ± SEM)

	Spontaneous activity (Hz)	
Masseteric-CFA grouped data	Baseline	21.1 ± 2.9; $F_{2.1,22.8}$ = 3.5, P = 0.045, n = 12
	Post-CFA 90 min	33.8 ± 5.3; t_{11} = 2.5, P = 0.028*
Masseteric-CFA "responders"	Baseline	18.0 ± 3.5; $F_{2.0,14.3}$ = 5.3, P = 0.019, n = 8
	Post-CFA 60 min	37.8 ± 7.4; t_7 = 4.2, P = 0.004*
	Dural-evoked "fast" neuronal responses (AP/Stim)	
Masseteric-CFA grouped data	Baseline	9.4 ± 0.4; $F_{3.0,32.8}$ = 10.7, P < 0.05
	Post-CFA 90 min	11.7 ± 0.5; t_{11} = 4.3, P = 0.001*
Masseteric-CFA "non-responders"	Baseline	9.8 ± 0.7; n = 4
	Post-CFA 90 min	11.6 ± 0.5
Masseteric-CFA "responders"	Baseline	9.3 ± 0.5; $F_{6,42}$ = 6.0, P = 0.008, n = 8
	Post-CFA 90 min	11.7 ± 0.7; t_7 = 3.1, P = 0.016*
	Dural-evoked "slow" neuronal responses (AP/Stim)	
Masseteric-CFA grouped data	Baseline	1.2 ± 0.2; $F_{2.0,20.4}$ = 6.5, P = 0.006, n = 11
	Post-CFA 90 min	2.9 ± 0.6, t_{10} = 3.3, P = 0.008*
Masseteric-CFA "responders"	Baseline	1.3 ± 0.2; $F_{1.8,12.9}$ = 7.2, P = 0.009, n = 7
	Post-CFA 90 min	4.4 ± 6; t_7 = 4.0, P = 0.005*

Note: AP/Stim = number of action potential spikes per stimulation.
*P < 0.05 significance from baseline.

3.6 | TMD-like (V3) pain increases the likelihood that CGRP will trigger, and exacerbates responses, of a migraine-like phenotype of dural-responsive trigeminocervical neurons

Co-morbid application of masseteric muscle-CFA with CGRP infusion caused a significant increase in spontaneous trigeminal neuronal firing over the 3 h (n = 10), which was significant from baseline after 60 min and remained significant beyond 3 h. There was a significant increase in firing in every neuronal population at least two time points, over the course of the 3 h, indicating that all were "responders" in this co-morbid condition. This change was accompanied by a significant increase in neuronal responses to dural stimulation for all "fast" neuronal discharges. This was specifically significant compared to baseline after 60 min that was maintained through 3 h. There was also a significant increase in responses for unitary discharges within the "slow" C-fibre range (n = 9). This was significant after 90 min that was also maintained through 3 h. For both "fast" and "slow" neuronal populations, the response after the combined treatment was greater than the sum of the CGRP and CFA groups alone. All data are summarized in Table 3 and Figure 5a–c.

In 9/10 neuronal populations, there was expansion of the cutaneous facial receptive field (examples in Figure 5d). There was also a hypersensitive neuronal response to cutaneous facial stimulation, with increased firing in response to innocuous brush and noxious pinch. The neuronal response to innocuous brush was significant after 45 min (Figure 5e), and the response to noxious pinch was significant after 90 min (Figure 5f), both of which were maintained through 3 h.

4 | DISCUSSION

Co-morbid migraine and TMD present various phenotypic features beyond being more likely than chance to be present in the same patient. TMD can itself cause secondary headache (Headache Classification Committee of the International Headache Society [IHS], 2018; Schiffman et al., 2012) and its presence can exacerbate existing headache symptoms, increase headache prevalence, especially migraine, and ultimately support the progression to chronic headache. Our data provide insights into the neural mechanisms related to the co-morbidity of intracranial headache, particularly migraine headache, with TMD, focusing on three major phenotypic features relevant to the clinical setting.

1. Does TMD-like pain mediate a headache-like neuronal response, per se? Correlating to the fact that TMD can itself cause intracranial headache, similar to migraine headache attributed to TMD (ICHD 11.7).
2. Does TMD-like pain increase the likelihood of a migraine-like neuronal response? Correlating to increased migraine prevalence when co-morbid.
3. Does the co-morbid condition exacerbate the migraine-like phenotype? Correlating to the fact that when co-morbid, migraine symptoms can be more severe, particularly cutaneous allodynia.

Using masseteric-CFA, a recognized approach to model myofascial TMD-like inflammation, and established electrophysiological methods used to study migraine-like mechanisms, this study demonstrates that modelling extracranial TMD-like muscle inflammation (similar to myositis) in male rats can produce neuronal activation and sensitisation of intracranial dural-responsive trigeminal neurons. This response is likely the neural correlate of the development of spontaneous intracranial throbbing head pain, alongside cutaneous allodynia and hyperalgesia, similar to migraine-like headache mechanisms. The response was observed in 2/3 neurons and was reversible, returning to baseline after 150 min, correlating with the recovery of spontaneous nociceptive grooming behaviours seen in mice (Romero-Reyes et al., 2015).

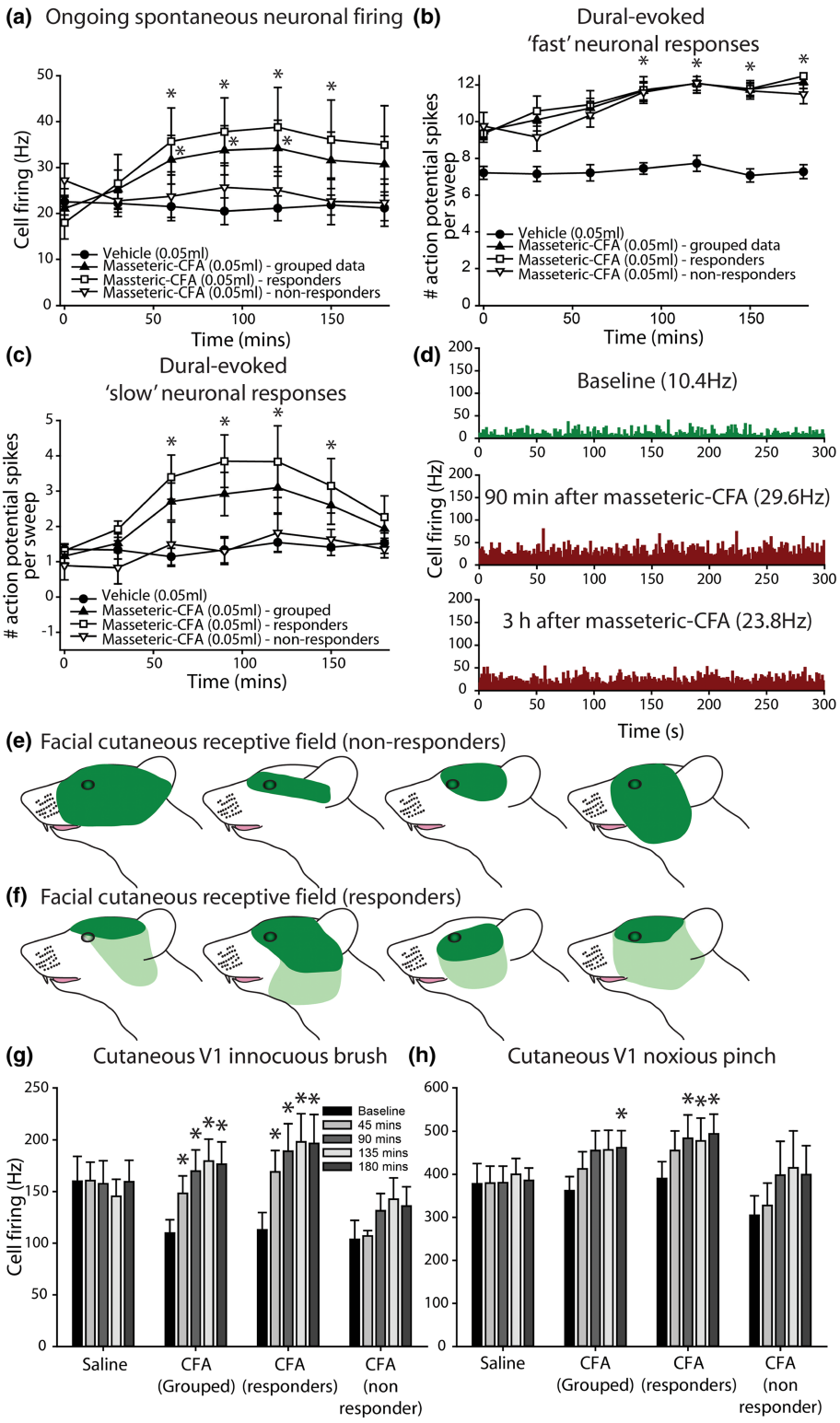

FIGURE 3 Masseteric muscle-CFA mediates reversible activation of central trigeminocervical neurons and neuronal hypersensitivity cranial somatosensory stimulation. Time course changes in (a) spontaneous trigeminocervical neuronal firing (action potentials per second [Hz]), (b) intracranial dural-evoked "fast" neuronal responses (3–20 ms or 3–30 ms range) and (c) unitary discharges within only the "slow" C-fibre latency range (20–80 ms) in response to masseteric muscle injection of complete Freund's adjuvant (CFA, 50 µl, i.m. $n = 12$). The data are expressed as mean ± SEM. Further, the data are grouped into all data (grouped) and those considered "responders" or "non-responders" to CFA. (d) Representative peristimulus time histograms from a single animal demonstrating ongoing trigeminocervical neuronal firing before CFA, and at 90 min and 3 h post infusion, in a "responder" animal. The numbers indicate the mean firing (spikes per second [Hz]) over the displayed time period, green neuronal firing indicates baseline and no change in responses, red neuronal firing indicates a significant increase in neuronal firing. Overall, both the grouped and "responder" data showed a reversible increase in ongoing trigeminal neuronal firing and hypersensitivity of "fast" and "slow" neuronal responses to dural-stimulation after CFA ($^*P < 0.05$ compared to baseline). In the "non-responders," there was no change in ongoing firing of "slow" neuronal responses, but they exhibited hypersensitivity of "fast" neuronal responses to CFA. Examples of cutaneous receptive fields in rats that were either (e) non-responders (no expansion) or (f) responders (expanded) to CFA. Dark green represents the original receptive field and light green is the expanded receptive field (including darker green region). Response magnitude to (g) innocuous and (h) noxious cutaneous stimulation of facial receptive field after CGRP. Only "grouped" and "responders" showed a delayed hypersensitive neuronal response to cutaneous stimulation after CFA. $^*P < 0.05$ statistically significance compared to baseline

Rats with an initial cutaneous receptive field that overlapped the V3 trigeminal innervation (region of CFA injection) were no more likely to have neurons as "responders" than if receptive fields were restricted to only the V1 cutaneous region. This supports the view that there is convergence of trigeminal neuronal projections from the extracranial V3 (and likely V2) structures, onto neurons that receive inputs from the intracranial V1-dural region, either onto the same, or adjacent neurons, in the trigeminocervical complex (Olesen, Burstein, Ashina, & Tfelt-Hansen, 2009). Noxious inflammation in the V3 region causes neuroplastic changes in synaptic communication altering the

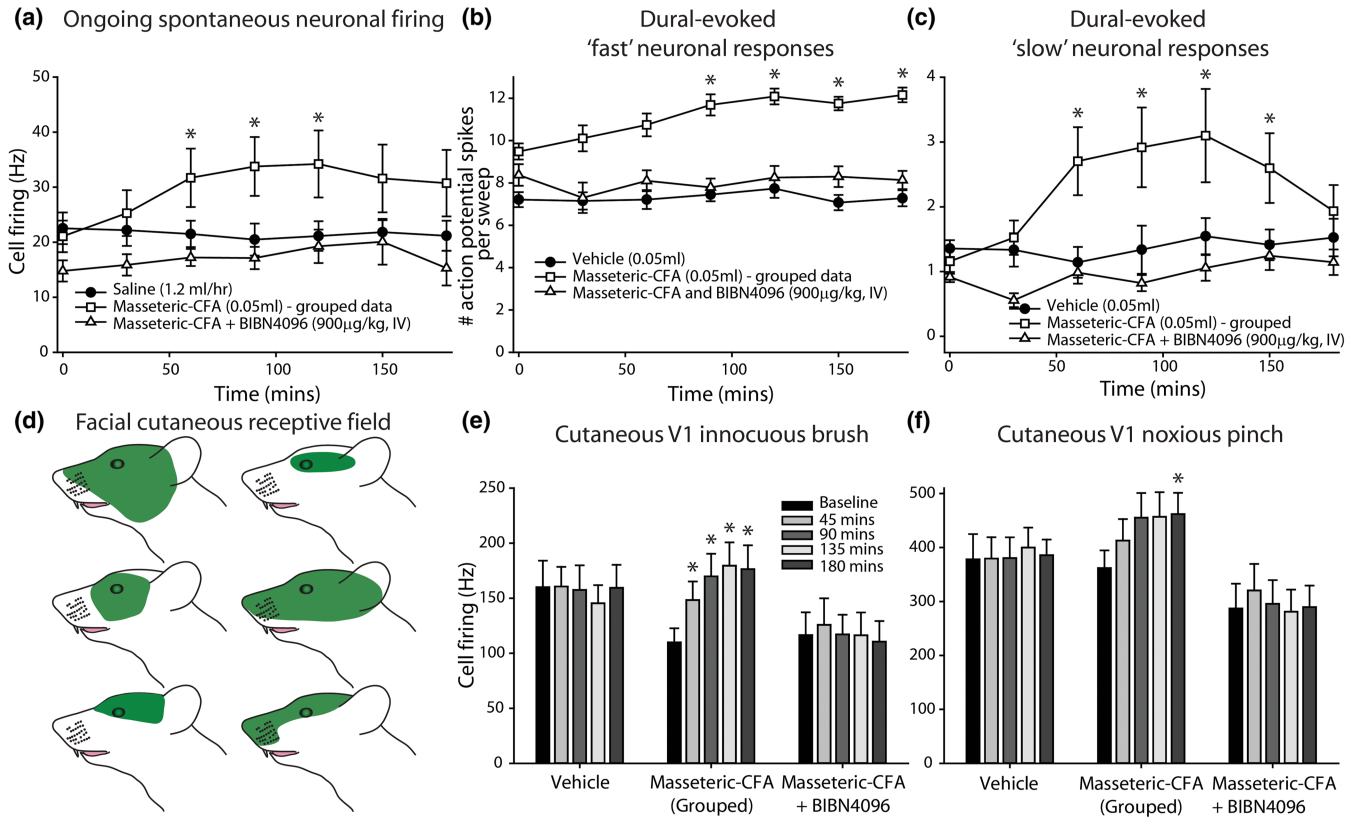

FIGURE 4 A selective CGRP receptor antagonist prevents the effects of masseteric muscle-CFA on dural-responsive trigeminocervical neurons. Time course changes in (a) spontaneous trigeminocervical neuronal firing (action potentials per second [Hz]), (b) intracranial dural-evoked "fast" neuronal responses (3–20 ms or 3–30 ms range) and (c) unitary discharges within the "slow" C-fibre latency range (20–80 ms). The data are expressed as mean ± SEM. (d) Examples of cutaneous facial receptive fields (dark green-original receptive field) in rats. Response magnitude to (e) innocuous and (f) noxious cutaneous stimulation of the facial receptive field. All data are in response to masseteric muscle injection of complete Freund's adjuvant (CFA, 50 µl, i.m.), or intravenous (i.v.) administration of a selective CGRP receptor antagonist, BIBN4096 (900 $\mu g \cdot kg^{-1}$, i.v.), followed by CFA. BIBN4096 (n = 12) prevented the increased activation of trigeminocervical neurons mediated by masseteric-CFA, and the hypersensitive neuronal responses to cranial somatosensory stimulation. Further, there was no expansion of facial cutaneous receptive fields. $^{*}P < 0.05$ represents a statistically significant difference compared to baseline

state of central trigeminocervical neurons, leading to their sensitisation, which outlasts the acute effects of the peripheral input. This can create the perception of hypersensitive activation of V1 intracranial-durally innervated trigeminal neurons; the neural correlate of intracranial-like headache, but without a direct intracranial stimulus or pathology. The reverse has previously been demonstrated, with intracranial-V1 dural inflammation leading to expansion of a neuronal receptive field to extracranial V2/V3 cutaneous trigeminal regions (Burstein, Yamamura, Malick, & Strassman, 1998). This previous work and our study provide support for the concept of cross-excitation between trigeminal regions at the level of central trigeminovascular neurons to explain the co-morbidity of migraine and TMD, although this convergence and cross-excitation could extend to mechanisms related to the peripheral trigeminal ganglion (Durham, 2016). Thus, TMD-like masseteric muscle pain can mediate an intracranial headache-like response and cutaneous facial allodynia, translating to observations in patients, as outlined by the International Classification of Headache Disorders (ICHD) and the International Classification of Orofacial Pain (ICOP).

We also demonstrate that these neuronal responses were prevented by pretreatment with a selective CGRP receptor antagonist. Previous data suggest that there are CGRP-mediated mechanisms in TMD-like pain. We have previously shown that CGRP antagonism attenuates nocifensive grooming behaviours and neuronal activity in the trigeminal system in response to masseteric-CFA (Romero-Reyes et al., 2015) and CGRP injection in the TMJ promotes cellular changes indicative of sensitisation (Cady et al., 2011). Our new data confirm this but also in the context of CGRP's involvement in mediating intracranial headache-like neuronal responses to masseteric-CFA. CGRP's role in the development of migraine headache and as a target for treatment is long established (Karsan & Goadsby, 2015). Together, this suggests that CGRP may have a crucial role as a molecular link within the trigeminal system in mediating the severe noxious responses related to this co-morbidity. Based on this link to both disorders we chose to use exogenous CGRP as our migraine-like approach, allowing us to specifically probe the impact of directly activating the CGRP pathway in a co-morbid model with TMD-like muscle pain.

TABLE 3 Summary of trigeminocervical neuronal data over 3 h, with responses at baseline and when significantly increased, in response to combined masseteric-CFA and CGRP (mean ± SEM)

	Spontaneous activity (Hz)	
Masseteric-CFA and CGRP	Baseline	22.2 ± 2.9; $F_{6,54}$ = 10.4, P < 0.05, n = 10
	Post-CFA-CGRP 60 min	34.6 ± 5.2; t_9 = 7.3, P = 0.001*
	Post-CFA-CGRP 3 h	42.3 ± 4.2; t_{11} = 3.1, P = 0.009*
	Dural-evoked "fast" neuronal responses (AP/Stim)	
Masseteric-CFA and CGRP	Baseline	8.0 ± 0.4; $F_{2.1,18.7}$ = 17.6, P < 0.05
	Post-CFA-CGRP 60 min	11.4 ± 0.7; t_9 = 4.6, P = 0.001*
	Post-CFA-CGRP 3 h	14.0 ± 1.2; t_9 = 5.0, P = 0.001*
	Dural-evoked "slow" neuronal responses (AP/Stim)	
Masseteric-CFA and CGRP	Baseline	1.3 ± 00.3; $F_{6,48}$ = 12.3, P < 0.05, n = 9
	Post-CFA-CGRP 60 min	1.8 ± 0.4; t_8 = 3.0, P = 0.017*
	Post-CFA-CGRP 3 h	3.3 ± 0.5; t_8 = 5.5, P = 0.001*

Note: AP/Stim = number of action potential spikes per stimulation.
*P < 0.05 significance from baseline.

Alone CGRP mediates activation and sensitisation of migraine-like dural and cutaneously innervated trigeminal neurons in a subset of rats (60%). This is likely the underlying neurophysiological mechanism for CGRP to trigger experimental migraine in patients (Hansen, Hauge, Olesen, & Ashina, 2010). The response to CGRP was delayed, with no effect at 60 min, but with significant migraine-like trigeminovascular neuronal responses from prolonged observations made over 3 h. This is similar to our previous studies using other exogenously administered experimental migraine triggers in preclinical models (Akerman et al., 2019; Akerman & Goadsby, 2015), where we believe the delay supports the lack of a role of the more immediate vascular changes and more likely an impact of CGRP on neural mechanisms. Further, CGRP receptor components are thought to be present on Aδ-fibres only and not C-fibre neurons (Eftekhari, Warfvinge, Blixt, & Edvinsson, 2013). This is further supported by the fact that a CGRP antibody selectively inhibited the responsiveness of trigeminal Aδ-fibre neurons, but not C-fibre neurons (Melo-Carrillo, Strassman, et al., 2017). Here, CGRP only caused hypersensitive neuronal responses to dural stimulation of Aδ-fibre trigeminal neurons, and not C-fibre responses, supporting these previous studies. Together, this validates CGRP as a translational experimental approach to study putative mechanisms in migraine-like headache.

Combining these two approaches we similarly observed sustained activation of dural-trigeminovascular neurons, with somatosensory hypersensitivity and expansion of cutaneous facial receptive fields beyond V1; signatures of sensitisation, but in every rat studied. It suggests that the presence of TMD-like (myositis) masseteric muscle pain/inflammation increases the likelihood of CGRP triggering a migraine-like neuronal response, translating to clinical features of this co-morbidity that when myofascial TMD is present it increases the prevalence of migraine in migraineurs (Goncalves et al., 2011). Furthermore, ongoing trigeminal firing and neuronal responses to somatosensory dural-intracranial stimulation were proportionally double that of CGRP alone and sustained beyond 3 h. This suggests in this co-morbid state the intracranial headache-like neuronal response is significantly exacerbated compared to CGRP given alone, again translating to a more severe migraine-like phenotype that patients' experience. Of note, this also occurred to C-fibre responses, which were prolonged beyond 3 h in the combined model and yet CGRP alone had no impact. It suggests that potentially already sensitized C-fibres (via their response to CFA) are affected by the overall neuronal environment, where sensitised Aδ-fibre responses are maintained beyond 3 h. However, sensitized Aδ-fibres alone cannot help recruit and sensitise non-responsive C-fibre neurons. Overall, this supports that this co-morbid preclinical neuronal profile translates to the phenotype present in migraineurs with TMD, supporting this combination approach as a relevant platform to study putative mechanisms related to this co-morbidity. It may also provide an opportunity to study therapeutic management protocols for TMD with headache disorders, and to screen for potential novel targets. While we anticipate other combinations, such as with nitroglycerin, are likely to be similarly successful, its limitation for our study is that it is known to cause the release of other noxious neuropeptides and inflammatory mediators, as well as CGRP, which would have limited our ability to dissect specific CGRP-related effects.

The mechanism and locus of action of CGRP in this co-morbidity is still not clear. It is likely that both peripheral and central neural actions are involved. CGRP receptor components are present at peripheral nerve endings, trigeminal ganglion cells, afferent projections and within the trigeminal nucleus (Eftekhari et al., 2015; Eftekhari & Edvinsson, 2011; Lennerz et al., 2008). Systemic injection of a CGRP receptor antagonist is more efficacious than local injection into the masseteric musculature when evaluating nociceptive responses to masseteric muscle-CFA (Ambalavanar et al., 2006). This suggests that actions on the neural projection are more relevant. The efficacy of CGRP antibodies in migraine therapy (Paemeleire & MaassenVanDenBrink, 2018), which are unlikely to get into the brain, strongly suggests that peripheral release of CGRP, via TMD-like pain, and activation of trigeminal ganglion cells is sufficient to alter the state of central neurons. However, other evidence suggests CGRP is involved in neurotransmission of trigeminocervical neurons to mediate an altered state of central neurons, such as central sensitisation, with CGRP receptor antagonists known to prevent this neurotransmission (Sixt, Messlinger, & Fischer, 2009; Storer, Akerman, & Goadsby, 2004). TMD-like pain may also mediate CGRP release at trigeminocervical neurons that also receive inputs from intracranial-dural projections, causing their sensitisation and provoking a migraine-like nociceptive phenotype.

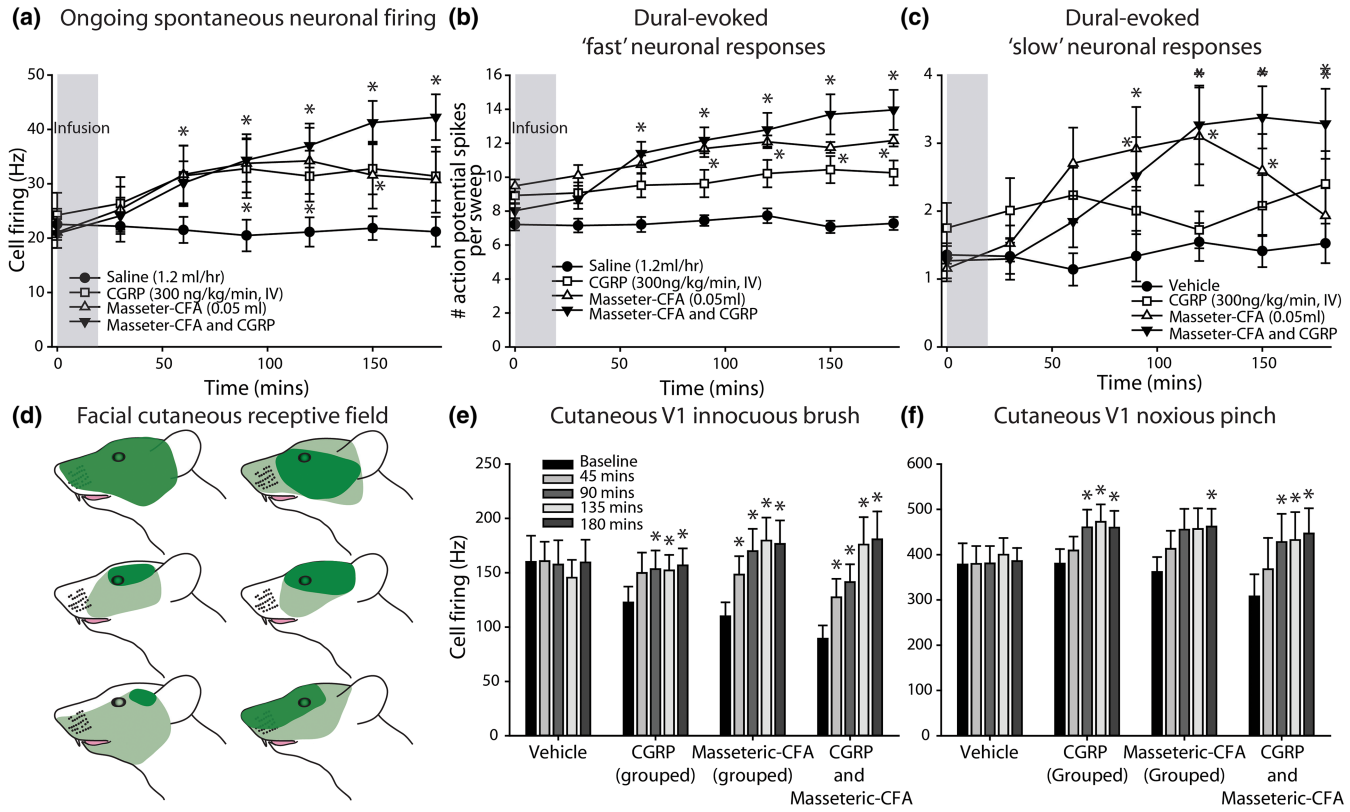

FIGURE 5 Masseteric muscle-CFA co-morbid with CGRP infusion exacerbates responses of dural-responsive trigeminocervical neurons to cranial somatosensory stimulation. Time course changes in (a) spontaneous trigeminocervical neuronal firing (action potentials per second [Hz]), (b) intracranial dural-evoked "fast" neuronal responses (3–20 ms or 3–30 ms range) and (c) unitary discharges within the "slow" C-fibre latency range (20–80 ms). The data are expressed as mean ± SEM. (d) Examples of cutaneous facial receptive fields (dark green-original receptive field) in rats, and their expansion after treatment (light green region, including darker region). Response magnitude to (e) innocuous and (f) noxious cutaneous stimulation of facial receptive field. All data are in response to either infusion of CGRP (CGRP for 20 min), masseteric muscle injection of complete Freund's adjuvant, or a combination of both. The combination (n = 10) treatment caused activation and hypersensitive neuronal responses in every animal tested, compared to only a subset of animals with single treatment. Further, dural-evoked responses were significantly exacerbated in the combination group compared to each group alone. Only one animal did not show expansion of a cutaneous facial receptive field. $^{*}P < 0.05$ represents a statistically significant difference compared to baseline

With our data, we can begin to dissect the mechanisms related to the clinical phenotype of this co-morbidity. A myofascial TMD-like pathology can mediate a migraine-like response via convergence of afferents onto dural trigeminal projections. As this increases the likelihood of a migraine-like response, an independent migraine-like pathology mediated either centrally (Goadsby et al., 2017) or through activation of meningeal nociceptors (Noseda & Burstein, 2013), causes further release of neurotransmitters, including CGRP, at the second-order synapse. These mediate a sustained sensitisation, resulting in a more prolonged and severe migraine phenotype, as demonstrated by the more severe migraine-like neuronal response.

With our overall interpretation of the data, we do add the caveat that these studies were conducted in male rats only. Therefore, we are cautious to not overinterpret our findings to responses in female rats, particularly given that migraine and TMD are both more prevalent in the women than men. That said, we would observe that previous studies in male rats have proven to be hugely translational with respect to understanding migraine and TMD mechanisms, as well as predicting therapeutic responses that translate to the clinic, for both men and women. Furthermore, studies that have sought to compare the responses of sexes in rodent models related to migraine mechanisms have found that, if anything, responses are exacerbated in females compared to males. Therefore, in summary, we demonstrate a preclinical approach with considerable translational overlap to the clinical features of co-morbidity of TMD and headache, particularly migraine. We believe it provides the opportunity to begin to dissect unique insights into the neural mechanisms involved in this co-morbidity and potentially factors that contribute to the most severe phenotypes of migraine. It offers a platform for potential future studies to dissect putative mechanisms of this co-morbidity, such as migraine chronicity. Finally, previous studies, and our new data support that targeting CGRP may be an efficacious approach to treat TMD alone, although to date no clinical trial studies have been conducted to validate this. However, we now also provide data to support the important role of CGRP in co-morbid mechanisms and therefore the CGRP pathway may also be a target as a monotherapy in the co-morbid condition and worthwhile exploring in clinical trials. Determining a strategy to more effectively

manage this co-morbidity would have an enormous public health impact on these patients, as well as having huge socio-economic implications.

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AUTHOR CONTRIBUTIONS

SA and MRR conceived and designed the study. SA and MRR performed all the experiments, SA analyzed all the data and wrote the first draft of the paper. MRR provided critical review of each draft. Both SA and MRR provided final approval of the manuscript.

CONFLICT OF INTEREST

S.A. reports personal fees from Amgen, Allergan, Novartis and GSK, and personal fees from Patent/Legal work in headache and orofacial pain, unrelated to this work. M.R.R. reports personal fees from Amgen, Allergan, Novartis, and GSK, and personal fees from Patent/Legal work in headache and orofacial pain unrelated to this work.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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